Structure identification for compound I separated and purified from taxoids-produced endophytic fungi (*Alternaria. alternata* var. *taxi* 1011)

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Abstract: Endophytic fungi are widely found in almost all kinds of plants. Many endophytic fungi can produce some physiological active compounds, which are same to or analog to those isolated from their hosts. Producing physiological active compounds through microbial fermentation can give a new way to resolve resource limitation and to find out alternative source. Through the methods of organic solvent extraction, thin layer chromatography (TLC) and column chromatography, compound I was isolated, purified from the liquid fermentation metabolites of the taxoids-produced endophytic fungi (*Alternaria. alternata* var. *taxi* 1011 Y. Xiang et LU An-guo) that was screened from the bark of *Taxus. cuspidata* Sieb.et Zucc.. Compound I was identified as one kind of taxoids type III, based on the analyzing results by using the methods of ultraviolet spectroscopy (UV), infrared spectroscopy (IR), mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). This study provides a completed method for separation and purification of the endophytic fungi as well as structure identification of its fermentation metabolite

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Introduction

The natural paclixtel extracted from the bark of *Taxus* spp. is a kind of diterpene of taxoids (Shi 1997; Jin *et al.* 1997; Zhou *et al.* 2000), according to its skeleton's character, whose structure can be classified to six kinds: ① 6/8/6 (I), ②5/7/6 (II), ③6/10/6 (III), ④6/5/5/6 (IV), ⑤6/12 (V), ⑥5/6/6 (VI). Among which, compounds of skeleton type I are the most common, and its amount occupies two-third of all the compounds of six types. The amounts of the rest five types occupy one-third (the amount of compounds of skeleton type II is second, compared with that of type I, the amount of the rest four is smaller). The paclixtel is mainly type I. It can promote polymerization of tubulins and inhibit depolymerization of tubulins, thus inhibiting the growth of tumor cells. In 1992 FDA approved that the paclixtel could be used to treat recurrent metastatic ovaries and enacted treatment standard of using together with cisplatin. In many countries, the paclixtel is also used to treat obstinate metastatic mammary cancer and has satisfactory effect (Zhang *et al.* 1997). However, the content of the paclixtel is very small in *Taxus* spp.. Although in recent years, some studies have been carried out on isolating and screening endophytic fungi that can produce paclixtel from *Taxus* spp., (Strobel, *et al.* 1996; Wang 1999; Wang 2000), further study is still needed about screening strains highly producing the paclixtel, about the methods of separation purification of the fermentation metabolites, about the structure skeleton's character of effective component and drug-action and so on. In this paper, according to the studies on conditions of isolation, screening and fermentation for *Taxus cuspidata* Sieb.et Zucc. endophytic fungi[*Alternaria. alternata* var. *taxi* 1011 Y. Xiang et LU An-guo, highly producing taxoids], (Xiang 2002), the authors separated and purified the taxoids produced by *Alternaria. alternata* var. *taxi* 1011 and identified its structure. The article summarized the methods of separation and purification for highly producing strain, as well as, structure identification methods of its fermentation metabolite.

Materials and methods

Sources of *Taxus* specimen

Alternaria.alternata (Fr.) Keissler var. *taxi* 1011 Y. Xiang et LU An-guo (highly producing taxoids) were collected from the bark of *Taxus cuspidate* Sieb. et Zucc. growing in the primeval forest of the Changbai Mountain Natural Reserve.

Liquid fermentation media

Seed medium: The glucose of 2g, 0.3-g peanut powder, $MgSO_4$ of 0.3 g, KH_2PO_4 of 0.3 g, NH_4Cl of 0.3 g, distilled water of 1000 mL were used, with natural pH. The sterilization was carried out at 115 °C for 15 min.

Fermentation medium: The optimization medium for strain fermentation was composed of glucose of 2.2 g,

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peanut powder 2.6 g, soybean powder 0.2 g, starch 2.2 g, peptone 0.48 g, yeast extract paste 0.5 g, $MgSO_4$ 1 g, KH_2PO_4 0.3 g, NH_4Cl 0.3 g. The distilled water was used for 1000 mL with natural pH. And the sterilization was at 121°C for 30 min

Reagents

Chemicals: the silica gel GF254 used was made in Qingdao Haiyang Chemical Co., Ltd., the silica gel H used was made in Qingdao Haiyang Chemical Co., Ltd., and vanillic aldehyde used was made in, Shenyang Deagent III factory. Others are analytical pure reagent.

Dilute bismuth potassium iodide: The bismuth subnitrate of 0.85 g adding acetic acid glacial of 10 mL was dissolved in the distilled water of 40 mL. Prior to use, 5-mL solution above, potassium Iodide solution of 5 mL and acetic acid glacial of 20 mL were added to the beaker, and then diluted to 100 mL with distilled water.

Potassium Iodide: Potassium Iodide of 16.5 g was dissolved by distilled water to 100 mL.

Instruments

The instruments used in the experiment are listed as follows:

LC-10AT High Performance Liquid Chromatography Instrument, SHIMANIU (Japanese);

SPD-10A Ultraviolet-visible Spectrophotometer, SHIMANIU (Japanese);

Spherisob C_{18} column (150 mm×40 mm), Physico-chemical Institute, Dalian;

MP- Miniature Melting Point Detector, Yanaco (Japanese);

UV-9100 -visible Spectrophotometer, Beijing Ruili Analysis Institute Co., Ltd.;

UV-III Multi-function Ultraviolet Analysis Instrument, Beijing Kaixing Telecommunications Equipment Institute;

UV-2201 Ultraviolet Scanner, SHIMANIU (Japanese);

IFS55 Infrared Scanner, BRUKER (America);

ARX300 Nuclear Magnetic Resonane Scanner, BRUKER (America);

Others are common Instruments.

Methods

Separation and purification of the fermentation metabolite of the strain

The fermentation metabolite of the strain were separated, purified and extracted using the methods of organic solvent extraction, thin layer chromatography (TLC) and column chromatography (Yao *et al.* 1996; Jin *et al.* 1997). The fermentation liquid of the strain was centrifuged at 3000 rpm per 10 min. The superior clarification liquid was concentrated to 1/10 of primitive volume. Concentrated liquid and precipitation with added ethyl acetate of equal volume were extracted three times respectively, then the ethyl acetate was merged, evaporated to dry, and solved with 5-mL 5-mL chloroform respectively, through big type silica gel column (36 cm×2 cm) with chloroform- methanol solution (100:1-1:1) for phase gradient elution. All portions of effluent were collected and separately volatilized to dry, after then, through the thin layer chromatography, the separation level and the site of compound I were detected. The useful effluents above were merged, evaporated to dry, and solved with 2-mL chloroform, through small type silica gel column (20 cm×1 cm) with chloroform- methanol solution (100:1-1:1) for phase gradient elution. The method is same to above. The useful effluents were collected for thin layer chromatography, through the method of useful effluents scrapping on the thin layer slate. At the last, the useful component was collected and the site of compound I was defined. The scraped silica gel was solved with methanol, dried, and recrystallized.

Extract could be detected by high performance liquid chromatography. The content of metabolite (compound I) could be determined by solving compound I in ethyl acetate and detecting the OD_{256} of solution with UV-visible Spectrophotometer. Since the absorption value of compound I was direct ratio to its concentration in a certain range, the OD_{256} value could be used to represent the content of compound I.

Structure identification of fermentation metabolite

The structure of compound I was identified by the methods of ultraviolet scanning, infrared scanning, mass spectrometry, nuclear magnetic resonane, and so on (Yao *et al.* 1996; Jin *et al.* 1997; Zhou *et al.* 2000) .

(1). Ultraviolet scanning: Solving the sample product in ethyl acetate and then detecting its UV absorption values with UV-2201 Ultraviolet Scanner.

(2). Infrared scanning: a small quantity of solid sample product was collected, grinded adequately and was pressed to tablet with KBr method, for detecting the infrared spectrum of sample product with IFS55 Infrared Scanner.

(3). Mass spectrometry: a small quantity of sample product solved in methanol was taken for detecting the proton charge proportion of its molecule and fragment peak with LCQ Mass Spectrometry Instrument.

(4). Nuclear magnetic resonane: Solving the sample product in deuterium, and then using TMS as inside marker to detect its 13C-NMR, 1H- NMR spectrum with ARX300 Nuclear Magnetic Resonane Scanner.

Results

Separation and purification of fermentation metabolite

A compound was separated, purified from the liquid fermentation metabolites of *Alternaria.alternata* (Fr.) *Keissler* var. *taxi* 1011, namely compound I. The compound I reacted with dilute bismuth potassium iodide, at first, it was violet, then, turned to orange-red, thus the compound I was identified as a kind of alkaloid.

Structure identification of the fermentation metabolite

Physical property of compound I

The appearance of compound I is buff snowflake powder. It is easy to be solved in methanol, ethyl acetate, and in chloroform, but not solved in water. Its melting point is from 148 °C to 152 °C. The optical activity is at $(\alpha)_D^{27}$+260 ° (MeOH).

Ultraviolet scanning

Ultraviolet Scanning showed that ultraviolet absorption peak of compound I is 256.2 nm, moderate absorption is 285.8 nm, and weak absorption is 354.8 nm. It is concluded that this compound has long chain conjugate system or dense cycle aromatic chromophore. It has at least 4-5 chromophore of mutual- conjugation because this compound is colored substance.

Infrared scanning

From Infrared scanning, the results show that compound I has –NH or –OH (3424.6cm^{-1}), -C=C (1644.3 cm^{-1}), lipo-carbon (2921.4 cm^{-1}, 1458.5cm^{-1}, 1369.2 cm^{-1}, 1179.5 cm^{-1}).

Mass spectrometry

Based on the result of mass spectrometry, the molecule-ion peak m/z of compound I is 636.0. The molecular weight of this compound I should be m/z-1 (635.0) because the authors ionize compound I with mass spectrometry instrument adopting the APCI technology (atmospheric pressure chemical ionization). Its secondary spectrometry was analyzed, and compound I had fragment peaks of 577.9 and 418.9 (m/z). The peaks are ethyl side chain having lost benzene cycle and skeleton side chain C-20 having lost benzene cycle.

^{13}C-NMR

According to detection result of ^{13}C-NMR and correlated literature (Zhou 2000), the compound I was defined as taxoids, its skeleton is 6/10/6 (type III), and chemical shift of carbon atom (Table 1, 2).

Table 1. Chemical shift of skeleton carbon of compound I in ^{13}C-NMR

No. of carbon	Chemical shift of compound I	Chemical shift of taxoids type III	Chemical shift of carbon	Chemical shift of compound I	Taxoids type III
C-1	47.8	47.4-51.4	C-11	132.6	132.4
C-2	69.3	68.4-72.0	C-12	135.6	135.9
C-3	34.6	34.9-35. 8	C-13	202.3	198.0-202.2
C-4	132.4	130.0-138.4	C-14	27.2	27.6
C-5	66.1	68.4-70.9	C-15	42.8	41.7-43.8
C-6	33.7	32.7	C-16	24.7	24.7
C-7	69.3	71.1	C-17	32.3	32.6
C-8	51.9	52.7-53.4	C-18	14.1	13.8-16.4
C-9	205.0	202.6-215.1	C-19	22.7	20.7
C-10	76.6	76.8	C-20	124.2	123.3-127.9

From the results above, the carbon chain skeleton of compound coincides with the carbon chain skeleton of taxiods.

^{1}H-NMR

According to the detection result of ^{1}H –NMR, the compound I has proton signals of δ 12.70 (m, H) and δ 12.34 (m, H) in low field. The structure of this compound has two brisk association hydrogen atoms. The proton signals of olefin were δ 7.85 and δ 7.82. The proton signals of olefin were also δ 7.10 and δ 7.26. The proton signals of δ 5.34 may be the proton signals of nitrogen.

Chemical structure of compound I

According to ultraviolet detection, vanillic aldehyde development, dilute bismuth potassium iodide (alkaloids developer) development and the above-mentioned spectroscopy, the chemical structure of compound I is one kind of taxiods type III. The compound I and taxiods type I are all taxiods. The chemical name of compound I is 2-oxygen ethyl-5- ethylamine-7, 10- dihydroxy-20-[4-2,6 (oxygen ethyl-benzene)-1,3-diene]-4,
20-diene-paclixtel-11-monoenoic-9, 13-diketon (Fig. 1).

Table 2. Chemical shift of side chain carbon of C-20 of compound I in ^{13}C-NMR

Carbon of side	Chain chemical shift	Carbon of side chain	Chemical shift
C-1'	19.5	C-8'	116.9
C-6'	162.0	C-13'	33.7
C-3'	117.5	C-10'	162.3
C-4'	139 2	C-11'	33.9
C-5'	122.8	C-12'	29.1
C-7'	130.1	C-14'	29.2

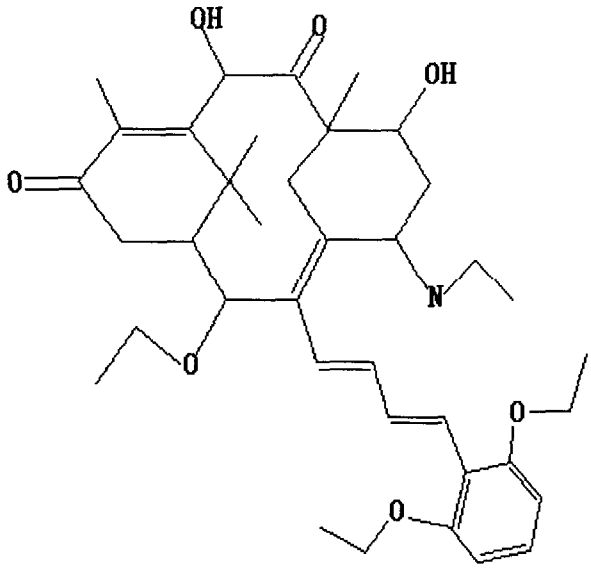

Fig. 1 Structure of compound I

Discussion

Extraction of the metabolite

Through organic solvent extraction, thin layer chromatography (TLC) and column chromatography, the compound I had been separated from the liquid fermentation metabolites of *Alternaria.alternata* (Fr.) *Keissler* var. *taxi* 1011 Y. Xiang et LU An-guo. During the whole procedure of extraction, the most capital procedure was the separation of column chromatography, including its fill-sample quantity, the proportion of eluent of silica gel column, the volume of the eluent of every proportion, and flow-rate of eluent and so on. These factors all can affect the result of separation.

Detection of HPLC

Through the detection of high performance liquid chromatography, the results showed that the percentile content of compound I was 98.95. After analyzing its detection conditions, it was found that the detected wave-length is 256 nm. Under the conditions, the absorption weak cannot be detected because there were no absorption substances or their detection values are smaller than normal values. So the authors inferred that the determination values of the percentile content of compound I were slightly higher than normal values.

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